HIV envelope induces a cascade of cell signals in non-proliferating target cells that favor virus replication

Claudia Cicala*†‡, James Arthos*†‡, Sara M. Selig*, Glynn Dennis, Jr.§, Douglas A. Hosack§, Donald Van Ryk*, Marion L. Spangler*, Tavis D. Steenbeke*, Prateeti Khazanie*, Neil Gupta*, Jun Yang§, Marybeth Daucher*, Richard A. Lempicki§, and Anthony S. Fauci*

*Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; and §Laboratory of Immunopathogenesis and Bioinformatics, National Institute of Allergy and Infectious Disease, National Institutes of Health, Frederick, MD 21702

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Certain HIV-encoded proteins modify host-cell gene expression in a manner that facilitates viral replication. These activities may contribute to low-level viral replication in nonproliferating cells. Through the use of oligonucleotide microarrays and high-throughput Western blotting we demonstrate that one of these proteins, gp120, induces the expression of cytokines, chemokines, kinases, and transcription factors associated with antigen-specific T cell activation in the absence of cellular proliferation. Examination of transcriptional changes induced by gp120 in freshly isolated peripheral blood mononuclear cells and monocyte-derived-macrophages reveals a broad and complex transcriptional program conducive to productive infection with HIV. Observations include the induction of nuclear factor of activated T cells, components of the RNA polymerase II complex including TFII D, proteins localized to the plasma membrane, including several syntaxins, and members of the Rho protein family, including Cdc 42. These observations provide evidence that envelope-mediated signaling contributes to the productive infection of HIV in suboptimally activated T cells.

HIV preferentially replicates in proliferating CD4$^+$ T cells (1). However recent evidence suggests that, *in vivo*, resting and suboptimally activated T cells may serve as targets for low-level productive infection in the absence of cellular proliferation (2–5). Infection in this manner may contribute to the establishment and/or maintenance of persistent viral reservoirs that currently prevent the eradication of virus. To productively infect suboptimally activated CD4$^+$ T cells, HIV must overcome post-entry barriers to replication (6–8).

DNA microarrays have been used to characterize the effect of HIV on target cell transcription (9, 10); in one microarray-based study, HIV Nef was shown to diminish barriers to viral replication by mimicking antigen-specific T cell proliferation signals (11, 12). It has been suggested that HIV gp120 also facilitates replication in suboptimally activated cells (12–15). Gp120 transduces near-simultaneous signals through CD4 (16), a component of the T cell receptor complex, and CCR5, a chemokine receptor (17–19). *In vivo* concentrations of gp120 (20, 21) fall within the range required to induce signaling *in vitro* (17–19, 22). To provide a more complete picture of the complex cascade of signals induced by gp120, we treated freshly isolated peripheral blood mononuclear cells (PBMCs) and monocyte-derived macrophages (MDMs) with an envelope derived from a CCR5-using virus and measured temporal changes in the levels of mRNA by using Affymetrix (Santa Clara, CA) U95A oligonucleotide microarrays that include probes encompassing ≈12,600 genes. In addition, we used a high-throughput Western blot analysis that allowed us to screen protein lysates with 800 monoclonal antibodies. The gp120 used was derived from JR-FL, a CCR5-tropic molecular clone obtained from a minimally passaged viral isolate (23). We used concentrations of envelope near or below that detected in the serum of infected patients (20).

Materials and Methods

Cells and Reagents. PBMCs were obtained by apheresis from normal volunteers followed by ficoll-hypaque isolation. Cells were resuspended in RPMI medium 1640/10% FBS. Donor PBMCs carrying one or two CCR5 Δ32 alleles were not used. MDMs were derived from elutriated monocytes cultured for 10 days in RPMI medium 1640/10%FBS/10% pooled human AB serum supplemented with granulocyte/macrophage colony-stimulating factor (GM-CSF; 10 units/ml). Gp120 was expressed and purified as described (17, 24). Briefly, a Chinese hamster ovary (CHO) cell line expressing a recombinant gp120 derived from the HIV molecular clone JR-FL (23) was cultured in hollow-fiber cartridges (Fibercell Systems, Frederick, MD). Protein was purified in three steps, employing metal-chelating, lectin, and size-exclusion chromatography. Protein concentrations were determined by absorbance at wavelength 280 nanometers. Protein was visualized by silver-stain and determined to be greater than 97% pure and endotoxin-free (LAL assay; BioWhittaker, Walkersville, MD). A mock protein prepared in an identical manner was derived from untransfected CHO cells and used as a mock.

gp120 Treatment of PBMCs and MDMs. Freshly isolated PBMCs and MDMs (1–5 × 10^7) per time point were incubated in 10% FBS/RPMI medium 1640 at 37°C during a time course ranging from 1 to 16 h at a concentration of 50 ng per 10^6 cells of gp120. Parallel cultures were treated with a mock protein preparation.

cRNA Preparation for Oligonucleotide Arrays. Total RNA from ≈1–5 × 10^7 stimulated or unstimulated cells per time point was extracted using the TRIZOL method (Life Technologies, Frederick, MD). Briefly, cell pellets containing 1 × 10^7 cells were lysed in 1 ml of TRIZOL and homogenized using a 1-ml syringe and a 19-gauge needle. Samples were layered with 200 μl of chloroform, inverted 15 times, and incubated on ice for 15 min. Lysates were centrifuged at 4°C for 15 min at 14,000 rpm and the aqueous phase transferred to a clean tube. RNA was precipitated with equal volumes of isopropanol for 15 min at room temperature. Precipitates were spun for 30 min at 4°C and 14,000 rpm. Pellets were washed twice with 70% cold EtOH, and dried at room temperature. RNA was resuspended in 50 μl of diethyl pyrocarbonate (DEPC) water, quantitated, and analyzed by denaturing gel electrophoresis to check purity. A total of 15 μg of RNA was used for microarray analysis. First- and second-strand DNA synthesis reactions were performed using the

Abbreviations: PBMCs, peripheral blood mononuclear cells; MDMs, monocyte-derived macrophages.

†C.C. and J.A. contributed equally to this work.

‡To whom reprint requests may be addressed at: 10 Center Drive, MSC 1876, Building 10, Room 11B13, Bethesda, MD 20892-1876. E-mail: ccicala@nih.gov or jarthos@nih.gov.

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a

1h PBMC	ttest/SAM	avg FC
Y09788 mucin 5	0.0211	8.0
AL050053 Homo sapiens mRNA	0.0827	6.3
AI377866 HNRPGT	0.0893	6.0
AI926989 Homo sapiens mRNA	0.0548	5.3
AF014794 TRAIL receptor 3	0.0419	4.8
AC004770 flap endonuclease 1	0.0537	4.7
AI954686 phakinin	0.0404	4.2
AL042599 Homo sapiens mRNA	0.0679	4.1
D84110 RNA-binding protein type 1	0.0738	3.9
U77845 TRAF interacting protein	0.0500	3.8
W84438 Homo sapiens mRNA	0.0882	3.7
AL080188 MT-protocadherin	0.0760	3.6
W28876 Homo sapiens mRNA	0.0665	3.5
AB028975 KIAA1052 protein	0.0793	3.4
L34408 Homo sapiens mRNA	0.0411	3.4
M69177 monoamine oxidase B	0.0467	3.2
L36069 K-channel alpha subunit	0.0764	3.0
AJ005890 JM1 protein	0.0168	3.0

5h PBMC	ttest/SAM	avg FC
AA021140 Homo sapiens mRNA	0.0596	6.0
AF030196 stannin	0.0393	4.8
U57094 RAS-Rerelated RAB-27	0.0121	4.3
U66346 cAMP phosphodiesterase 4C	0.0563	3.9
AB020675 Contactin associated 2	0.0609	3.8
AF035620 BRCA1 associated protein	0.0310	3.6
AI393342 Homo sapiens mRNA	0.0364	3.5
Y14153 beta-transducin repeat	0.0499	3.4
U02478 AF-6	0.0088	3.3
AL096748 Homo sapiens mRNA	0.0256	3.2
D26121 splicing factor 1	0.0053	3.1
AF038009 tyrosylprotein sulfotransferase1	0.0279	3.0
AB016899 HGC6.1.1	0.0463	3.0

PBMC all time points grouped	ttest/SAM	avg FC
U47931 G-protein beta-3 subunit	0.0191	6.2
M20469 clathrin light-chain b	0.0114	5.9
AL050053 Homo sapiens mRNA	0.0420	5.4
AI377866 HNRPGT	0.0120	5.1
Y09788 Mucin 5	0.0454	4.6
AB007887 KIAA0427 gene	0.0442	4.5
AF035620 BRCA1 associated protein	0.0593	4.4
AF050078 growth arrest-specific 11	0.0747	4.1
M65066 cAMP-dependent protein kinase	0.0553	3.9
D84110 RNA-binding protein type 1	0.0293	3.7
D26121 splicing factor 1	0.0270	3.5
X63359 UDP glycosyltransferase 2-B10	0.0454	3.4
AF040628 ectodermal dysplasia 1	0.0162	3.4
MAX dimerization protein (MAD)	0.0106	3.3
M69177 monoamine oxidase B	0.0459	3.2
U02478 AF-6	0.0277	3.1

16 PBMC	ttest/SAM	avg FC
M76446 adrenergic alpha-1D receptor	0.0559	16.5
X82540 activin beta-C chain	0.0075	7.8
U47931 Human G-protein beta-3	0.0274	7.8
M92432 Guanylate cyclase 2D	0.0642	6.4
U70426 Regulator of G-protein signalling 16	0.0293	6.2
S65761 IgG1 kappa	0.0445	6.1
U02478 AF-6	0.0357	5.7
U68418 aminotransferase 2	0.0935	5.7
Protein Phosphatase Inhibitor Homolog	0.0500	4.9
AB018279 vesicle glycoprotein 2a	0.0075	4.8
AF043978 transcription factor (PAX4)	0.0888	4.7
U16126 Kainate-selective glutamate receptor 2	0.0244	4.6
Y07595 transcription factor TFIIH	0.0574	4.5
U65402 G protein-coupled receptor 31	0.0500	4.5
U16720 interleukin 10 (IL10)	0.0450	4.3
X16354 Biliary glycoprotein I	0.0857	4.3
U63973 rhodopsin kinase	0.0803	4.3
AC003030 KIAA0892 protein	0.0588	4.2
M28130 interleukin 8 (IL8)	0.0675	4.2
Y07909 Epithelial membrane protein 1	0.0827	4.2
X16316 vav oncogene	0.0215	4.1
L19871 Activating transcription factor 3	0.0541	3.9
M19301 Lipoamide acyltransferase	0.0718	3.9
X54489 GRO1 oncogene	0.0316	3.9
U43959 adducin 2	0.0563	3.9
S66431 RB binding protein 2	0.0836	3.8
AB000905 H4 histone	0.0752	3.8
X12433 putative transmembrane protein	0.0277	3.8
AL080132 Homo sapiens mRNA	0.0169	3.7
U94902 CD44 (CD44)	0.0262	3.6
M80335 protein kinase alpha	0.0843	3.6
AF000297 NK-2 homolog 8	0.0367	3.4
AL049758 pacsin 2	0.0048	3.2
M22488 bone morphogenetic protein 1	0.0744	3.2
AF025409 zinc transporter 4	0.0181	3.2
D86331 matrix metalloproteinase 15	0.0814	3.1
U31116 beta-sarcoglycan	0.0825	3.0

b

3h MDM	ttest/SAM	avg FC
W26524 Protein phosphatase 4	0.0078	10.4
D83174 heat shock protein 47	0.0893	8.1
AF038847 36 kDa FK506 binding protein	0.0469	7.0
U25265 MAP kinase kinase 5 (MEK5)	0.0971	6.8
AF016917 GABA A receptor, delta	0.0072	6.0
AF042083 BH3 interacting domain death agonist	0.0750	5.1
AB028975:KIAA1052 protein	0.0432	5.0
X52773 Retinoid X receptor, alpha	0.0897	4.9
S83374 Glutamate transporter II	0.0032	4.1
Y13467 PPAR binding protein	0.0379	3.9
J04809 cytosolic adenylate kinase (AK1)	0.0758	3.8
AB006622 KIAA0284 protein	0.0539	3.7
AI040181 KIAA0645 gene	0.0526	3.7
AJ007041 trithorax homologue 2	0.0465	3.5
AL109698 Homo sapiens mRNA	0.0329	3.5
U06452 melanoma Ag (MART-1)	0.0116	3.4
D79989 centaurin, gamma 1	0.0009	3.4
J05582 mucin 1	0.0455	3.3
AB014589 Homo sapiens mRNA	0.0136	3.3
AF000148 polyadenylation protein CSTF-64	0.0169	3.3
U70862 Nuclear factor I/B	0.0877	3.3
AF034102 nucleoside transporter	0.0066	3.3
AF007138 NDRG family, member 4	0.0299	3.2
U57796 zinc finger protein (LD5-1)	0.0573	3.2
U94190 Huntingtin-associated protein	0.0091	3.2
U79248 Homo sapiens mRNA	0.0978	3.2
AF073920 Regulator of G-protein signalling 6	0.0315	3.0

10h MDM	ttest/SAM	avg FC
L37127 RNA polymerase II	0.0511	14.4
X63749 Rod transducin alpha	0.0567	13.7
AL050370 Homo sapiens mRNA	0.0880	8.2
M20812 kappa-immunoglobulin	0.0122	7.7
L32164 zinc finger protein	0.0117	7.1
AL050374 Homo sapiens mRNA	0.0678	6.3
M99438 transducin-like enhancer of split 3	0.0334	6.1
X69950 Wilms tumor associated protein	0.0872	6.1
AI208485 hemoglobin, zeta	0.0672	6.0
AF049498 sodium channel	0.0718	5.9
Z50194 pleckstrin homology-like domain	0.0967	5.4
AB011117 KIAA0545 protein	0.0957	5.1
AB029037 trophinin	0.0931	4.7
D64137 Cdk-inhibitor KIP2	0.0770	4.6
Estrogen Receptor	0.0938	4.4
W26700 inorganic phosphate cotransporter	0.0573	3.9
U38260 islet cell autoantigen ICAp69	0.0106	3.8
AI677689 KIAA0685 gene	0.0773	3.8
W28983 Homo sapiens mRNA	0.0039	3.8
X04430 interleukin 6 (IL-6)	0.0954	3.7
M36634 vasoactive intestinal peptide	0.0256	3.6
AF015257 G protein-coupled receptor 30	0.0283	3.6
X07523 complement factor H	0.0385	3.6
AI762248 Homo sapiens mRNA	0.0400	3.4
L14812 RBL1 Retinoblastoma-like 1 (p107)	0.0449	3.4
U95090 Kidney/liver proline oxidase 1	0.0211	3.3
X79510 protein-tyrosine-phosphatase D1	0.0335	3.2
AI765053 Homo sapiens mRNA	0.0570	3.2
L12701 Human engrailed protein (EN2)	0.0527	3.2
AF029343 Protocadherin 17	0.0322	3.1
AB013924 Lysosomal-associated protein 3	0.0250	3.1
X65962 cytochrome P-450IIC	0.0690	3.0
X99947 axonemal dynein 9	0.0025	3.0

6h MDM	ttest/SAM	avg FC
U89896 casein kinase I gamma 2	0.0404	12.6
AA442560 Phorbolin-like protein	0.0570	12.5
AF082559 ADP-ribose polymerase	0.0144	11.0
AI249721 Homo sapiens mRNA	0.0697	6.9
X13967 Leukemia inhibitory factor	0.0856	6.7
U21090 DNA polymerase delta	0.0023	5.2
AF029343 protocadherin 17	0.0956	5.2
Homeotic Protein Hpx-5	0.0404	5.2
AB006684 autoimmune regulator	0.0854	4.9
AI700753 MAPK-activated protein kinase 5	0.0765	4.6
AB002294:KIAA0296 gene	0.0695	4.6
W29091 thyroid hormone receptor	0.0279	4.4
U91985 DNA fragmentation factor-45kd	0.0297	4.3
U65092 Cbp/p300-interacting transactivator	0.0433	4.2
W26787 Homo sapiens mRNA	0.0180	4.1
AF016052 zinc finger pr. ZNF191	0.0016	4.1
Y11392 mitochondrial protein	0.0107	3.9
M30185 cholesteryl ester transfer	0.0990	3.7
AC004597 olfactory receptor	0.0851	3.7
AF004327 angiopoietin-2	0.0078	3.6
AB018299 KIAA0756 protein	0.0053	3.3
U01062 IP3-receptor, type 3	0.0648	3.3
AC004262 Egf-like	0.0311	3.2
AF036973 HCGIV-6 protein	0.0387	3.2
M96740 Nescient helix loop helix 2	0.0102	3.2
AF073482 myotubularin related protein 7	0.0307	3.1
AC004381 Homo sapiens mRNA	0.0581	3.0
M92303 calcium channel beta-1 subunit	0.0552	3.0

MDM all time points grouped	ttest/SAM	avg FC
M28130 interleukin 8 (IL8)	0.0960	17.1
M21121 RANTES	0.0534	16.5
U82987 Bcl-2 binding component 3	0.0818	7.1
M17017 interleukin 8 (IL8)	0.0874	6.9
M17589 tyrosine hydroxylase	0.0731	5.5
U19523 GTP cyclohydrolase I	0.0323	5.0
AF016917 GABA A receptor, delta	0.0364	4.9
M64936 retinoic acid-inducible retroviral RNA	0.0537	4.6
AB006684 autoimmune regulator	0.0331	4.6
X04430 interleukin 6 (IL-6)	0.0722	4.1
M20812 kappa-immunoglobulin	0.0060	4.0
X66079 Spi-B transcription factor	0.0025	4.0
M38258 retinoic acid receptor gamma	0.0534	3.9
M21121 RANTES	0.0720	3.9
AF062739 GSK-3 binding protein FRAT2	0.0842	3.8
W27286 ElaC homolog 2	0.0278	3.8
AF042083 BH3 interacting domain death agonist	0.0034	3.7
U62801 protease M mRNA	0.0201	3.6
AI814466 Homo sapiens mRNA	0.0345	3.5
AF029343 protocadherin 17	0.0488	3.4
X69950 Wilms tumor associated protein	0.0584	3.4
AB002308 KIAA0310 gene	0.0151	3.4
AJ011896 Nef-associated factor 1	0.0535	3.3
AA019936 nucleolar autoantigen NO55	0.0423	3.3
L34041 glycerol-3-phosphate dehydrogenase 1	0.0424	3.3
AF102544 Molybdopterin synthase sulfurylase	0.0136	3.1
AI700753 Homo sapiens mRNA	0.0424	3.1
L37112 arginine vasopressin receptor 1B	0.0420	3.0

Fig. 1. Induction of genes in response to gp120 treatment. A list of genes, which were determined by SAM to be significantly modulated in response to JR-FL gp120, was generated. A subset of that list that includes those genes most up-regulated is listed by time point for PBMCs (*a*) and MDMs (*b*). Responses were also evaluated by SAM independent of time, and a subset of those genes that were most up-regulated is reported in the list termed ''all time points.'' Accession number and definition are included for each gene. avg FC, the fold change relative to PBMCs or MDMs treated with a mock protein preparation. Genes are ranked by descending fold change. Results represent the average of four donors in PBMCs or three donors in MDMs.

Table 1. JR-FL gp120-induced expression of genes previously associated with HIV

Induced gene	Cell type	GenBank accession no.	Ref. and/or PubMed ID no.
Naf-1	PBMC, MDM	AJ011896	11, 12
RANTES	PBMC, MDM	M21121	35
MIP-1α	PBMC	D90144	35
MIP-1β	PBMC	J04130	35
NFATc	PBMC, MDM	U08015	11, 28, 29
IL8	PBMC, MDM	M28130	33
MGSA (Gro-α)	PBMC	X54489	34
IL10	PBMC	U16720	26
IL1β	PBMC	M15330	26
TAFII-28	PBMC	X83928	11
c-jun	PBMC	J04111	10027715
c-myc	PBMC, MDM	V00568	11208609
JKN2	MDM	U09759	9045910, 9403476
RNA polymerase II	MDM	L37127	11
LcK	MDM	M36881	8887682, 7953537
STAT2	PBMC	U18671	15
IFN-γ	MDM	L07633	26
TGFβ	PBMC	X02812	26
vav	PBMC	X16316	10394361, 9582304
p53	MDM	X02469	11313714
sCD44	PBMC	U94902	8345206
70Kd HSP1	MDM	M11717	11, 11727548
NFkB	MDM	M58603	15, 1380488

Genes determined by SAM analysis to be induced by JR-FL gp120 that have previously been reported to be modulated by HIV infection and/or gp120 signaling are listed along with the associated GenBank accession no. and literature citation.

Superscript Choice System (Life Technologies) followed by *in vitro* transcription (Enzo Diagnostics) using biotin-labeled dNTPs. The resulting biotin-labeled cRNA was quantitated and

Table 2. Incidence of descriptive categories associated with genes modulated by gp120

Descriptive term	*P* value
Cytokine	8.6E-06
Growth factor	3.1E-05
Ligand	1.6E-04
Inhibitor or repressor	2.7E-04
Chemokine	3.5E-04
Response to bacteria	4.0E-04
Cell motility	5.3E-04
Antiviral response protein	1.3E-03
Response to biotic stimulus	1.3E-03
Chemotaxis	1.4E-03
Stress response	1.8E-03
Cell migration/motility	4.3E-03
Defense response	9.0E-03
Viral replication	9.7E-03
Cell proliferation	2.3E-02

A SAM-derived list of genes modulated in both PBMCs and MDMs by gp120 was analyzed for significant association with descriptive categories that distinguish this list from the rest of the genes represented on the GENECHIP. Descriptive categories were derived from Gene Ontology and Proteome At-A-Glance classification systems. Descriptive categories that were significantly over-represented in the list that includes all of the genes modulated by HIV envelope at all time points, were ranked by *P* value (one-tailed Fisher Exact test). Shown are the top ten over-represented terms and five other highly represented terms relevant to the theme of viral replication.

Table 3. Cytokines and chemokines induced by JR-FL gp120 in PBMCs and MDMs

	Accession no.	Cell type	HIV replication	Ref./PubMed ID no.
Cytokine				
IL1β	M15330	PBMC ↑	↑	26
IL3	M20137	PBMC ↑	↑	26
IL6	X04430	MDM ↑	↑	26
IL10	U16720	PBMC ↑	↑ ↓	26
IL13 precursor	U31120	MDM ↑	↓	26
IL15 precursor	AF031167	MDM ↑ PBMC ↓	↑	9533656
IFN β 2A	X04430	MDM ↑ PBMC ↑	↓	26
IFN γ	L07633	MDM ↑	↑ ↓	26
INF ω 1	X58822	MDM ↑		
TNF α	X02910	MDM ↑ PBMC ↑	↑	26
VEGF	X58822	PBMC ↑		
VEGF B precursor	U48801	PBMC ↓		
LIF (IL6 family)	X13967	MDM ↑ PBMC ↓	↑	8207643
Chemokine				
GRO-α	X54489	PBMC ↑	↑	34
GRO-γ	M36821	PBMC ↑		
NAP2	M54995	MDM ↓		
IL8	M28130	MDM ↑ PBMC ↑	↑	33
IP-10	X02530	PBMC ↓		
MIP-1β	J04130	PBMC ↑	↑ ↓	35
MIP-1α	D90144	PBMC ↑	↑ ↓	35
RANTES	M21121	MDM ↑ PBMC ↑	↑ ↓	35
MIP-3α	U64197	PBMC ↑		

Cytokines and chemokines that were differentially expressed in either PBMCs and/or MDMs at any individual time point as determined by SAM analysis are listed along with their GenBank accession no. The cell type in which differential expression was detected is listed. Increased mRNA expression is denoted by ↑, and decreased mRNA expression by ↓. Effect on viral replication, based on published; literature is also listed by arrows.

analyzed for purity on a 2% agarose Tris acetate EDTA (TAE) gel. cRNA samples were then fragmented and prepared for hybridization to Affymetrix Human Genome U95A oligonucleotide arrays according to protocols specified by the manufacturer.

Western Blot Analysis. Immunoblot analysis of proteins was carried out as described (www.translab.com/shtml). Briefly, PBMCs were treated with gp120 as described above and proteins were lysed in 10 mM Tris (pH 7.4)/1 mM Na+orthovanadate/1% SDS followed by sonication and clarified by centrifugation. Gels used were 16 × 16 cm, 5–15% gradient SDS-polyacrylamide, 1-mm-thick. A gradient system was used so a wide size range of proteins could be detected on one gel. Four hundred micrograms of protein was loaded in long well across the entire width of the gel. This translates into ≈15 μg per lane on a standard 25-well gel. The gel was then run at constant milliamps. Subsequently the gel was transferred to Immobilon-P membrane (Millipore, Bedford, MA) overnight at 200 mA. After transfer, membranes were blocked for 1 h with 5% milk. Subsequently, the membrane was inserted into a Western blotting manifold that isolates 45 channels across the membrane. In each channel, different complex antibody cocktails were added and allowed to hybridize for 1 h. Following staining, the membranes were washed and hybridized for 30 min with secondary goat anti-mouse horseradish peroxidase (HRP). All antibodies were mouse monoclonal. Membranes were washed and developed with SuperSignal West Pico (Pierce).

Statistical Determination of Significant Differential Expression. A Significant Analysis of Microarrays (SAM; ref. 25) algorithm was used to determine significant differential expression after extensive prefiltering of genes. The prefilter was established as follows. The expression value, termed average difference (Avg. Diff.), was derived using the Affymetrix software program MAS 4.0. The Avg. Diff. of genes <10 were truncated to 10. The difference between mean Avg. Diff. between comparison groups was set at >30. Mean Avg. Diff. between comparison groups was set at >1.4-fold or <−1.4-fold, and the significant difference of a Student's t test between the comparison group was set at $P < 0.1$. Only those genes that generated lists from SAM after 5,000 randomizations and with a 90% median false discovery rate equal to zero were defined as differentially expressed.

Results

Six Hundred Genes Modulated by HIV-1 Envelope in PBMCs and MDMs. Fresh PBMCs (four donors) were treated with gp120 for 1, 5 and 16 h, whereas MDMs (three donors) were treated for 3, 6, and 10 h. In PBMCs, >9,000 genes were expressed of the ≈12,600 genes represented on the microarray whereas in MDMs, >8,900 genes were expressed. To identify genes that were modulated in a significant manner, we used a recently developed statistical method termed SAM (25). This method was designed specifically to analyze large numbers of biological responses (≈10,000) typical of microarray data. Using SAM we identified ≈600 genes (≈300 for PBMCs and ≈300 for MDMs) that were significantly modulated in response to envelope treatment. From that list we extracted subsets of genes that were up-regulated to the greatest degree (Fig. 1 *a* and *b*). We report these responses at each of the time points analyzed (1, 5, and 16 h for PBMCs; 3, 6, and 10 h for MDMs). Because individual donors may modulate a given gene at different time points, we also identified genes that were significantly modulated irrespective of the time of the response (Fig. 1 *a* and *b*; all time points). Although many of the genes we identified are of unknown function or have never been associated with HIV, our analysis identified at least 23 genes that have previously been associated with HIV replication and/or envelope signaling (Table 1). This finding indicates that the system and strategy that we used was sufficiently sensitive and reliable

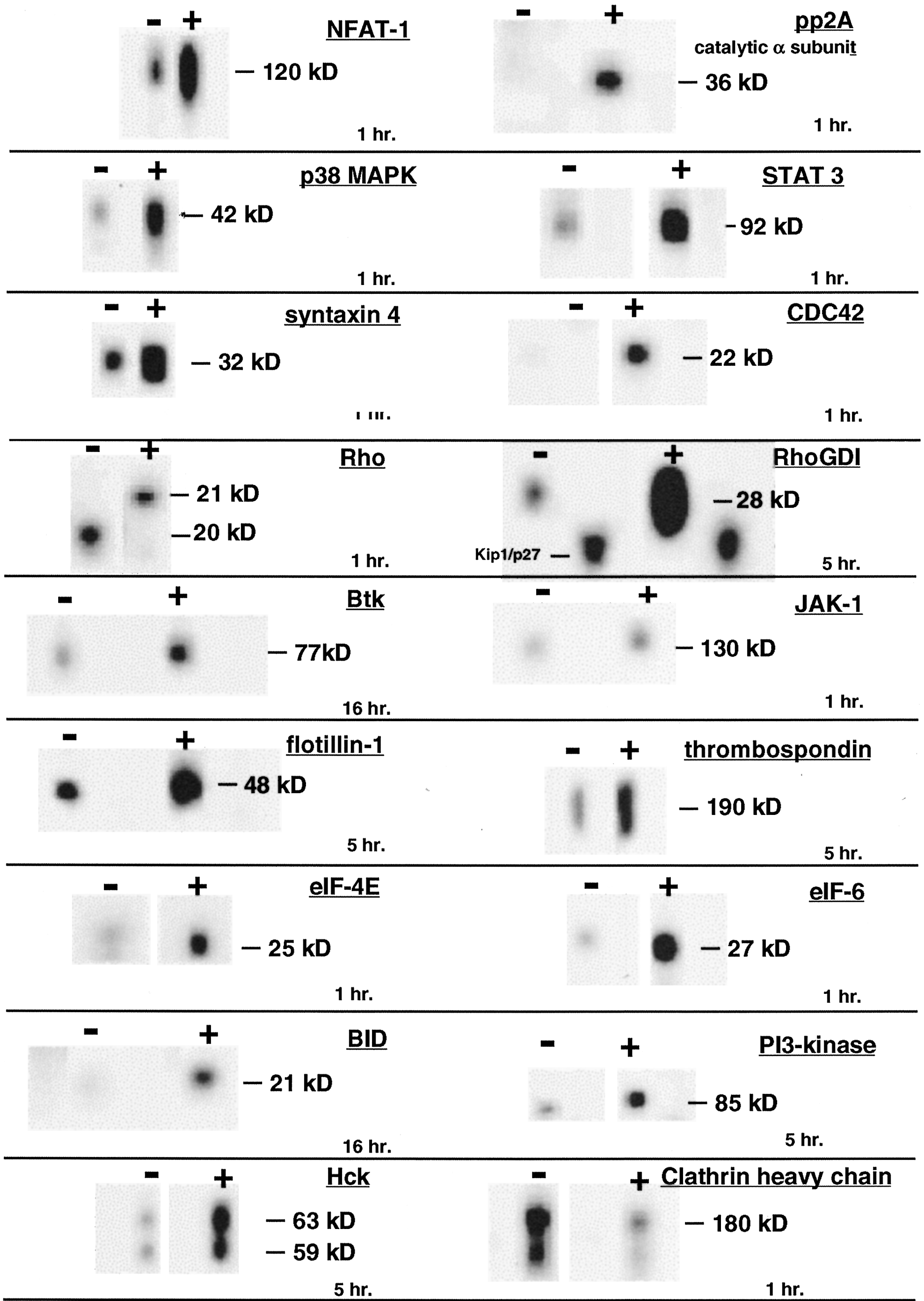

Fig. 2. Western blot analysis of gp120-treated PBMCs. Freshly isolated PBMCs were treated with JR-FL gp120 for 1, 5, or 16 h. Lysates were analyzed by Western blot analysis with a collection of ≈800 mAbs or sera (www.translab.com/TOC.shtml). Selected results are displayed.

to identify relevant responses. We chose to focus our current analysis on genes likely to influence viral replication.

The Term *Cytokine* Ranked Highest Among Descriptive Categories Modulated by gp120. To assess the potential of the ≈600 differentially expressed genes to influence the replication of HIV, we used a newly developed literature-mining algorithm (D.A.H. and R.A.L., unpublished work). This algorithm utilizes a set of descriptive categories that includes all of the descriptive terms in the Gene Ontology and Proteome At-A-Glance classification systems found in the Locus-Link reports (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/locuslink). We identified descriptive categories of genes that were over-represented in our list of differentially expressed genes, relative to all 8,443 annotated genes represented on the U95A microarray. In this manner, we generated an index with corresponding significance values. Envelope-induced genes encompassed 353 descriptive categories and among those categories the term *cytokine* achieved the highest degree of significance and was among the most frequently associated with gp120-modulated genes (Table 2). HIV replication is strongly influenced by cytokines *in vivo* (1), and thus this result is highly consistent with the hypothesis that envelope-mediated cell signaling plays a role in viral replication. Other relevant terms, including *cell proliferation* and *viral replication*, also occurred with a high degree of significance (Table 2).

We observed increased transcription of twelve cytokine messages (Table 3). TNF-α, IL1-β, and IL3 all enhance HIV replication (26) and all were up-regulated in PBMCs. In MDMs, four cytokines that enhance HIV replication, TNF-α, IL6, IL15, and IFN-γ, were up-regulated. Only two cytokines that suppress viral replication, IL13 and IFN-β, were induced.

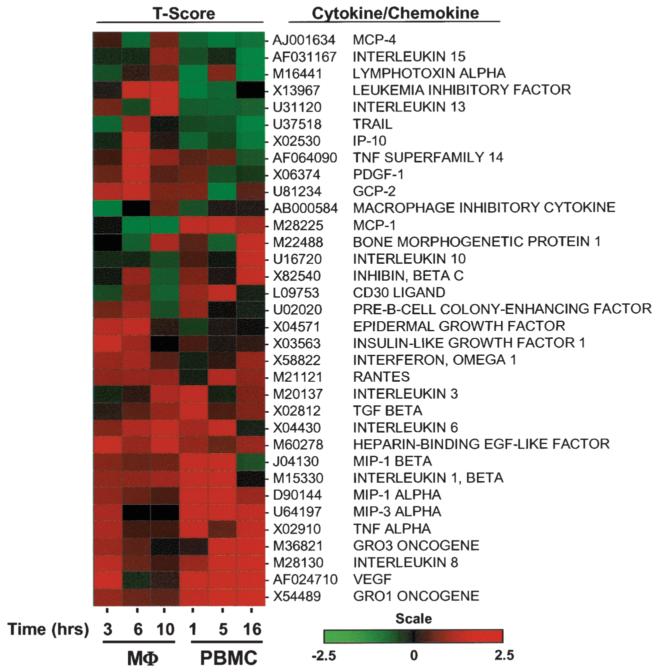

Fig. 3. Hierarchical cluster analysis of the genes included in the category "Cytokine/Chemokine" that are induced by gp120. Genes included in this that were significantly differentially expressed in macrophages (three donors; Mϕ) or PBMCs (four donors) following treatment with gp120 were clustered (SPOTFIRE software package, Spotfire Inc., Somerville, MA; hierarchical algorithm) according to t score values derived after performing 5,000 random permutations of the expression data by using a paired test comparing treated versus untreated samples (SAM software package). Changes in gene expression relative to untreated PBMCs and MDMs are indicated by a color scale in which the color red indicates up-regulation of transcription and green indicates down-regulation. Changes in gene expression for three different time points in both MDMs and PBMCs are represented.

This pattern of cytokine induction, although complex, favors viral replication.

Increased Expression of the Transcription Factor NFAT (Nuclear Factor of Activated T Cells). An increase in NFATc message was observed in both PBMCs and MDMs (Table 1). In addition, Western blot analysis demonstrated that gp120 induced a pronounced increase in NFAT-1 protein expression (Fig. 2). The NFAT family of transcription factors plays a central role in the transcriptional regulation of inflammatory cytokines and other genes central to immune responses (27). Two NFAT recognition elements reside in the HIV long terminal repeat (LTR) (28), and both NFAT-1 and NFATc induce the replication of HIV in primary cells (28–30). Furthermore, NFATc promotes the productive infection of HIV in the absence of $CD4^+$ T cell proliferation (29). This occurs as a consequence of two activities: NFAT facilitates reverse-transcription of the HIV genome (29), and it strongly induces transcription from the HIV LTR (28). Because NFAT acts both directly on the HIV transcription and indirectly through increased cytokine expression, we regard its induction as particularly significant to HIV replication. Although this is the first report of gp120 inducing NFAT expression, it has previously been shown that NEF induces NFATc transcription (11). Interestingly, the α subunit of protein phosphatase 2A (PP2A), which activates NFAT (31), was also up-regulated (Fig. 2).

Relevant Transcription Factors and Kinases Induced by HIV-1 Envelope. Other transcription factors and protein kinases, including c-jun, JNK, MEK, p38 MAPK, STAT, and JAK, all of which participate in antigen-specific T cell activation (14, 15, 32), were induced (Figs. 1 and 2, Table 1). We observed increased transcription of two components of the transcription-elongation factor complex, TFII H and TFII D (Fig. 1, Table 1). We also observed a large increase in the transcription of RNA polymerase II (Fig. 1, Table 1). In addition to NFATc, NEF also induces RNA polymerase II, TFII D, and a third component of the transcription-elongation complex, CDK9 (11). Because envelope-mediated signaling occurs at a very early step in HIV infection of target cells, and NEF is among the first proteins expressed, we suggest that gp120 and NEF may act synergistically in driving transcription from the HIV LTR in suboptimally activated cells.

gp120 Induced Five Chemokines and Modulates Chemokine-Related Signal Transduction. Five chemokines, IL8, GRO-α, GRO-γ, MIP1α, MIP1β, and RANTES, were up-regulated in response to gp120 (Figs. 1 and Fig. 3, Tables 1 and 3). IL8 and GRO-α enhance HIV replication (33, 34). GRO-γ is closely related to GRO-α and is also likely to enhance viral replication. Although MIP1α, MIP-1β, and RANTES are generally viewed as inhibitory chemokines, they only block entry of R5 viruses at very high concentrations (35). It is of note these same chemokines enhance viral replication at postentry steps of the viral life cycle (35–37). A common paradigm in gene expression analysis holds that genes that are regulated similarly function in a coordinated manner (38). In this regard, cluster analysis of genes included in the descriptive category "cytokine/chemokine" illustrate the simultaneous induction of Gro-α, IL-8, MIP-1α, and MIP1-β, which are all up-regulated within 5 h of treatment with envelope (Fig. 3).

Intracellular factors related to chemokine signaling and cell trafficking were also induced. We observed a marked increase in the expression of both RhoGDI and Cdc42, and an apparent modification of Rho (Fig. 2). Rho and Cdc42 are members of the Rho family of small guanine nucleotide-binding proteins that participate in lymphocyte trafficking by controlling the formation of focal adhesion complexes (39). We and others have previously demonstrated that gp120 induces the phosphorylation of FAK (40) and Pyk-2 (18), two components of focal adhesion complexes. We suggest that gp120-mediated induction of chemokines has a net effect that favors viral replication directly through the stimulation of target cells and indirectly through the recruitment of target cells to sites of active viral replication.

gp120-Mediated Modulation of a Group of Genes Involved in Membrane Fusion. Several genes associated with membrane fusion were induced by gp120. We found the increased expression of syntaxin-4 by gp120 within the first hour after treatment to be intriguing (Fig. 2). Induction of other syntaxin family members was observed at the level of mRNA. Syntaxin-7 was induced in PBMCs, whereas syntaxins 6 and 11 were induced in MDMs (data not shown). Syntaxins are members of a family of integral membrane proteins referred to as SNAREs (41). These proteins mediate membrane fusion in a compartment-specific manner. They play a central role in the fusion of intracellular vesicles with the plasma membrane (41). It has been suggested that syntaxins participate in the controlled exocytosis of cytokines from intracellular vesicles on T cell activation (42), a process consistent with gp120-induced cytokine and chemokine secretion. Of note, syntaxin-mediated membrane fusion is mechanistically similar to HIV-envelope-mediated fusion (43). Finally we note the induction of flotillin-1 (Fig. 2), a protein that is enriched in lipid rafts (44), which are membrane structures that facilitate fusion of HIV with plasma membranes of target cells (45).

Discussion

HIV-1 pathogenesis is regulated by a complex interaction between viral and cellular factors. A better understanding of these complex interactions will aid in the development of new classes of therapeutic agents designed to inhibit HIV-1 replication. We have demonstrated that CCR5-specific HIV gp120 induces the transcription and expression of factors that provide a conducive environment for HIV replication in resting or suboptimally activated PBMCs.

We used high-density oligonucleotide microarrays and high-throughput Western blotting to characterize the complex response of PBMCs and MDMs to gp120-mediated signaling. By microarray analysis, ≈300 genes were reproducibly modulated in both PBMCs and MDMs. This represents ≈3% of the total number of expressed messages detected by the Affymetrix U95A chip in our system. Changes in the expression of these genes may reflect both direct and indirect responses to envelope signaling. Presently we cannot distinguish between CD4-mediated versus CCR5-mediated signals.

The rapid induction of cytokines and chemokines at early time points is likely to initiate a cascade of responses. Using a literature-mining algorithm, we found genes related to cytokines and chemokines among the most highly represented groups of genes responding to gp120, an observation that underscores the potential impact that envelope-mediated signaling can impose on the transcriptional and translational programs of the human immune system. HIV replication *in vivo* is controlled by cytokines and chemokines, and the majority of those induced by gp120 enhance viral replication. In addition, gp120-induced dysregulation of cytokines and chemokines may contribute to immune dysfunction and HIV pathogenesis (1). Cytokines and chemokines that were modulated by gp120 were further analyzed by a cluster analysis program. By grouping genes that were similarly regulated in time, we observed the coordinated induction of a set of chemokines, all of which are reported to enhance viral replication. In addition, these chemokines may further enhance infection by recruiting lymphocytes to sites of viral replication

Intracellular factors including kinases and transcription factors associated with antigen-specific T cell activation were also induced. We observed an increase in the expression of NFATc and NFAT-1, factors that may be critically important in promoting viral replication. These observations support the hypothesis that signal transduction by the viral envelope contributes to the replication of HIV in resting or suboptimally activated cells. Of note, it has been suggested that such activity may contribute to the preferential transmission of R5 tropic HIV (32) and our data are consistent with this hypothesis. Interestingly, other viral gene products, notably NEF, also appear to promote the expression of factors favorable to viral replication (11). We suggest that these activities of gp120 and NEF increase the susceptibility of target cells to productive infection and may contribute to the low-level replication of HIV at sites set apart from activated T cells, thus avoiding immune surveillance. Replication in this manner may contribute to the establishment and maintenance of viral reservoirs, the persistence of which presents an obstacle to highly active antiretroviral therapy (HAART)-mediated eradication of HIV.

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